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Single Nanoparticle Plasmonic Spectroscopy for Study of Charge-Dependent Efflux Function of Multidrug ABC Transporters of Single Live *Bacillus subtilis* Cells

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Abstract

Multidrug membrane transporters can selectively extrude a wide variety of structurally and functionally unrelated substrates, and they are responsible for ineffective treatment of a wide range of diseases (e.g., infection and cancer). Their underlying molecular mechanisms remain elusive. In this study, we functionalized Ag NPs (11 nm in diameter) with two biocompatible peptides (CALNNK, CALNNE) to prepare positively and negatively charged Ag-peptide NPs (Ag-CALNNK NPs^{+ζ}, Ag-CALNNE NPs^{-4ζ}), respectively. We used them as photostable plasmonic imaging probes to study charge-dependent efflux kinetics of BmrA (ABC) membrane transporter of single live *Bacillus (B.) subtilis* cells. Two strains of the cells, normal expression of BmrA (WT) or devoid of BmrA (ΔBmrA), were used to study the charge-dependent efflux kinetics of single NPs upon the expression of BmrA. The NPs (1.4 nM) were stable (non-aggregated) in a PBS buffer and biocompatible to the cells. We found the high dependent accumulation of the intracellular NPs in both WT and ΔBmrA upon the charge and concentration of NPs. Notably, the accumulation rates of the positively charged NPs in single live WT cells are nearly identical to those in ΔBmrA cells, showing independence upon the expression of BmrA. In contrast, the accumulation rates of the negatively charged NPs in WT are much lower than in ΔBmrA, showing high dependence upon the expression of BmrA and suggesting that BmrA extrude the negatively charged NPs, but not positively charged NPs, out of the WT. The accumulation of positively charged NPs in both WT and ΔBmrA increases nearly proportionally to the NP concentration. The accumulation of negatively charged NPs in ΔBmrA, but not in WT, also increases nearly proportionally to the NP concentration. These results suggest that both negatively and positively charged NPs enter the cells via passive diffusion driven by concentration gradients across the cellular membrane, and BmrA can only extrude the negatively charged NPs out of the WT. This study shows that single NP plasmon spectroscopy can serve as a powerful tool to identify single plasmonic NPs and to probe the charge-dependent efflux kinetics and function of single membrane transporters in single live cells in real time.

Graphical Abstract

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INTRODUCTION

The ABC membrane transporters (efflux pumps) exist in all living organisms and form one of the largest protein families.^{1–3} They are involved in transport of a wide variety of structurally and functionally unrelated substrates (e.g., sugars, lipids, amino-acids, proteins, or xenobiotics). Extrusion of antibiotics or chemotherapeutic agents out of cells (e.g., bacteria or tumor cells) by the multidrug (multi-substrates) ABC membrane transporters leads to ineffective treatment of infection and cancer, which underscores the importance of understanding their efflux function in order to design more effective therapy.^{4–5}

Though the ABC membrane transporters can extrude a wide variety of substrates, they share a common modular architecture that includes four core domains: two transmembrane domains (TMD) with variable sequence and topology and two nucleotide-binding domains (NBD) with conserved sequences.^{6–9} Studies have showed that TMDs define the substrate binding-sites and form the transport passageway for substrates to cross the membranes, while the NBDs bind and hydrolyze ATP to power the transporters, which leads to extrude the substrates out of the cells against the concentration gradients across the cellular membrane, named as efflux function.^{6–7, 10–11}

One of the most intriguing questions about the multidrug ABC membrane transporters is how the structurally similar membrane transporters could selectively extrude a wide variety of structurally unrelated substrates.^{6–7, 10–11} We hypothesize that the specific conformations of membrane transporters might be assembled to selectively extrude a given pump substrate as they encounter with the substrate, underscoring the importance of characterization of their efflux functions in single live cells in real-time. Though X-ray crystallography and cryo-TEM are the primary workhorses to depict the structures of membrane transporters at the atomic resolution, none of them can provide real-time dynamic insights into how the pump proteins specifically interact with substrates and assemble membrane transporters to selectively transport the substrates.^{6–9, 12} Furthermore, crystallization of transmembrane proteins is always very challenging.

Radioisotopes (^{14}C and ^3H) and fluorophores (e.g., rhodamine 123, Fluo-3, Hoechst dyes) have been widely used as probes to study efflux kinetics of multidrug membrane transporters in both bacterial and mammal cells.^{13–20} These conventional probes have generally been used to study ensemble accumulation kinetics of substrates in bulk cells. Notably, individual membrane transporters and single live cells act distinctively and their efflux kinetics are unsynchronized. Thus, it is essential to study the efflux function of single membrane

transporters in single live cells in real time, in order to offer sufficient sensitivity to detect rare and individual membrane transport kinetics, and to directly measure the efflux rates of individual pump substrates, which would have been masked and undetectable by ensemble study. We have used fluorophore (EtBr) as an imaging probe and thin-layer total-internal reflection fluorescence microscopy and spectroscopy to study efflux kinetics of single membrane transporters of single live cells in real-time.¹⁷ However, single fluorophores or radioisotopes themselves do not possess distinctive size-dependent physicochemical properties that could be used to measure their sizes *in situ* in real time. Therefore, these conventional probes themselves cannot serve as various sized pump substrates for the study of size-dependent efflux function of single membrane transporters in single live cells.

Noble metal nanoparticles (e.g., silver nanoparticles, Ag NPs) show distinctive plasmonic properties, which highly depend upon their sizes, shapes, dielectric constants, and surrounding environments.^{21–28} Individual Ag NPs possess high Rayleigh scattering, and can be imaged and characterized, under illumination of a halogen lamp, using dark-field optical microscopy and spectroscopy (DFOMS).^{25–26, 29–38} Furthermore, we have demonstrated that we can use size-dependent LSPR (localized surface plasmon resonance) spectra and size-dependent scattering intensity of single NPs to quantitatively determine the sizes of single NPs in solution, in single live cells and single living embryos in real time, and we can use single Ag NPs as photostable nanometer sized optical probes to study the size-dependent efflux kinetics of multidrug membrane transporters in single live cells in real time, and to image single molecules of interest on/in single live cells for a desired period of time with both nm spatial and ms temporal resolutions.^{25, 29–36, 39–43}

In our previous studies, we have used size-dependent LSPR spectra of the noble metal NPs to study transport dynamics of single membrane transporters (MexAB-OprM) in single live cells (*Pseudomonas aeruginosa*, Gram negative bacterium), and to determine the transformation of pore sizes of their cellular membranes induced by antibiotics (e.g., aztreonam, chloramphenicol) in real time.^{30–32, 44} We have also used size-dependent LSPR spectra of the noble metal NPs to study the dependence of efflux kinetics of single BmrA membrane transporters in *B. subtilis* (a Gram-positive bacterium) upon the substrate size and the cellular expression of BmrA.^{29, 43} We have constructed two new strains of *B. subtilis*, BmrA fused with EGFP via its C- or N- terminus (Ct-BmrA-EGFP or Nt-BmrA-EGFP), and found that both strains retain the efflux function of BmrA.^{18, 29}

Studies have showed that some ABC membrane transporters can only extrude either positively or negatively charged substrates, while other ABC transporters can extrude both positively and negatively charged substrates.^{3, 45–52} The charges of amino acids at given locations of TMDs might play a key role in selection of given types of charged substrates.^{3, 45–52} Thus, the study of charged-dependent efflux function of membrane transporters offers the possibility to address how the structurally similar membrane transporters could selectively extrude a wide variety of structurally unrelated substrates. In this study, we synthesized and characterized positively and negatively charged Ag-peptide NPs with nearly identical sizes (11.5 ± 2.9 nm; 11.3 ± 2.5) and studied charge-dependent efflux function of BmrA in two strains of *B. subtilis* (WT with normal expression of BmrA and BmrA with non-expression of BmrA), aiming to demonstrate that the NPs can serve as powerful

imaging probes to study charged-dependent efflux function of single membrane transporters in single live *B. subtilis* cells. Notably, *B. subtilis* has widely served as a model organism to study efflux function of multidrug ABC membrane transporters, and 78 ABC transporters have been identified in *B. subtilis*.^{53–55} To our knowledge, it remains unknown whether the BmrA possesses charged-dependent efflux function.

EXPERIMENTAL SECTION

Reagents and Supplies

Sodium citrate (99%), AgClO₄ (99%) and NaBH₄ (98%) were purchased from Sigma Aldrich, and live/dead bac^{Light} viability and counting assay was purchased from Invitrogen. Peptides, Cys-Ala-Leu-Asp-Asp-Glu (>98%) and Cys-Ala-Leu-Asp-Asp-Lys-methyl ester (>98%), were purchased from Celtek Peptides. All reagents were used as received. The de-ionized (DI) water (18 MΩ, Barnstead) was used to prepare solutions and rinse glassware.

Synthesis and Characterization of Ag-peptide NPs

We reduced AgClO₄ (0.1 mM in DI water) with a freshly prepared ice-cold solution of sodium citrate (3 mM) and sodium borohydride (10 mM) under stirring overnight to prepare the colloidal Ag NPs (11 nm in diameter), and filtered the solution using 0.22 μm filters.^{30, 41} We added PEG-20 (0.05% w/v) into the colloidal Ag NPs and stirred the solution for 45 min. We added each type of peptides (CALNNK or CALNNE in a PBS buffer, 0.5 mM PBS buffer with 1.5 mM NaCl, pH 7.0) into the solution (20 mL colloidal Ag NPs) drop-wise under shaking.^{56–57} We continuously shook the solution at 4°C for 12 h to achieve the SN2 replacement reaction of –SH group of the cysteine of peptides with citrates adsorbed on the surface of the NPs to prepare Ag-CALNNK⁺ and Ag-CALNNE[–] NPs.⁵⁸

We washed the Ag-peptide NPs three times with the DI water using centrifugation to remove excess chemicals involved in synthesis, resuspended the NPs in the PBS buffer and characterized the NP concentrations, the LSPR spectra and images of single NPs, and sizes and charges of NPs using UV-vis spectroscopy (Hitachi U-2010), dark-field optical microscopy and spectroscopy (DFOMS), high-resolution transmission electron microscopy (TEM) (JEOL, JEM-2100F), and dynamic light scattering (DLS) (Nicomp 380ZLS particle sizing system), respectively.⁵⁸

Our DFOMS is equipped with a dark-field optical microscope with a dark-field condenser (oil 1.43–1.20, Nikon) and a 100× objective (Nikon Plan fluor 100× oil, iris, SL. N.A. 0.5–1.3, W.D. 0.20 mm) with a depth of field of 190 nm, a CCD camera (Micromax, Roper Scientific) and Multispectral Imaging System (MSIS, Nuance, CRI).^{31–32, 34, 37–38} The MSIS is an integrated system of a liquid-crystal-tunable-filter (LCTF) with CCD camera (SonyICX 285).^{35, 38, 40, 42, 59–60} We have used DFOMS-MSIS to simultaneously acquire dark-field plasmonic spectra and images of massive amount of individual NPs, which enables high-throughput spectral characterization of LSPR spectra of single NPs. We have fully described the designs and applications of our DFOMS for real-time imaging and spectroscopic characterization of single NPs in solutions, single live cells and embryos, and for single molecule detection (SMD).^{25, 29–42, 44}

Cell Lines and Cell Culture

We purchased WT (BmrA, with normal expression level of BmrA) *B. subtilis* from Bacillus Genome Stock Center (BGSC), and received BmrA (previously named as *yvcC* or *bmrA*, a mutant strain that is devoid of the *bmrA*) from J. M. Jault.²⁰ We pre-cultured the cells in an Erlenmeyer flask (250 mL) containing 20 mL of L-broth (LB) medium (1% tryptone peptone, 0.5% yeast extract, and 0.5% NaCl, pH = 7.2) in a shaker (Lab-line Orbit Envivon-Shaker) (150 rpm, 37°C) for 12 h. We then cultured the cells (WT or BmrA) in the LB medium for another 8 h. We harvested the cultured cells using centrifugation (Beckman Model J2-21 Centrifuge, JA-14 rotor, at 7500 rpm, 23°C, 10 min), and washed them with the PBS buffer three times. We re-suspended them in the buffer, adjusted the final cell concentration to OD_{600 nm} = 0.1, and used it for the entire study.

Real-time study of efflux function of membrane transporters in bacterial cells has always been conducted in PBS buffer, but not in cell culture medium.^{14–15, 18–19, 29, 61} The previous studies have showed that the bacterial cells in the PBS buffer retain their efflux function over hours.^{14–15, 18–19, 29, 61} Notably, the bacterial cells suspended in the PBS buffer retain their cellular growth stage and cellular function over hours, and their number remains the same over time.^{14–15, 18–19, 29, 61} Therefore, in order to use the same growth stage and number of the cells over the duration of each experiment (2 h), it is essential to study the accumulation rates of the NPs in the cells that are suspended in the PBS buffer, but not in the cell culture medium which would lead to the cell growth and creation of the cells with various growth stages and functions. As we reported previously, we characterized the efflux function of BmrA in live cells by real-time measuring the accumulation rates of Hoechst dyes (Hoechst 33342) in WT and BmrA using fluorescence microscopy.^{18, 29}

Imaging of Single Ag-Peptide NPs Inside and Outside of Single Live Cells

We incubated the cell suspension (OD_{600 nm} = 0.1) with 0.7 and 1.4 nM Ag-CALNNK NPs^{+ζ} or Ag-CALNNE NPs^{−4ζ} in the PBS buffer for 2 h. We started the timer to record the incubation time as we added the NPs into the cell suspension. We sampled the mixture into a freshly prepared microchamber every 20 min and imaged single live cells with the NPs at ~15 cells/min over every 20 min using DFOMS. This approach enabled us to image massive amount of cells (1800 cells) at single cell and single NP resolution for each sample and to gain sufficient statistics for study of the accumulation kinetics of NPs in bulk cells at single cell resolution. Distinctive plasmonic properties (colors) of single Ag NPs enabled us to use their LSPR spectra to identify single NPs over cellular membrane, cellular vesicles or debris, which appear white under dark-field illumination. We determined the number of individual intracellular NPs and plotted them versus time to study accumulation rates of single NPs in the cells over time (accumulation rate = slope of the plot over each given duration).

Real-time Imaging of Viability of Single Cells

After the 2 h incubation, we characterized the viability of the cells using live/dead BacLight bacterial viability and counting assay.⁶² We imaged the single cells using dark-field optical microscopy and epi-fluorescence microscopy, and counted the green fluorescence cells (peak wavelength of fluorescence spectra of SYTO9, λ_{max} = 520 nm) and red fluorescence

cells (peak wavelength of fluorescence spectra of propidium iodide, $\lambda_{\text{max}} = 610 \text{ nm}$) as live and dead cells, respectively.

Data Analysis and Statistics

For characterization of sizes, shapes, and LSPR spectra of single Ag-CALNNK NPs^{+ζ} or Ag-CALNNE NPs^{-4ζ} using TEM and DFOMS, we imaged a minimal number of 100 NPs per measurement, and repeated each experiment three times. Thus, a minimal number of 300 NPs was characterized for each type of the NPs. We acquired ten representative images of each cell suspension incubated with 0.7 or 1.4 nM Ag-CALNNK NPs^{+ζ} or Ag-CALNNE NPs^{-4ζ}, every 20 min over 2 h to study accumulation rates of intracellular NPs in single live cells in real-time. This approach allowed us to image a minimal number of 100 single live cells per measurement during every 20 min for 2 h. Thus, 600 single live cells were studied over 2 h for each measurement. We repeated each experiment three times. Thus, 1800 cells were studied for each sample over 2 h, which enabled us to gain sufficient statistics for study of accumulation rates and efflux kinetics of BmrA in bulk cells at single cell resolution.

We presented total number of the cells (300 cells) for all three measurements at the center of each given duration (20 min), but not average of three measurements (100 cells) with standard deviations. The reason that we did not use the standard deviation is because such standard deviation differs from the conventional standard deviation, which is used to determine the precision of the repeated measurements only. Notably, individual cells are distinctive and could have various accumulation and efflux kinetics. Therefore, standard deviations for single cell measurement would include not only the precision of three repeated measurements, but also variation of efflux and accumulation kinetics of single cells. In other words, the conventional standard deviation of repeated measurements only represents the precision of measurements, and hence it is not suitable to describe single cell measurements.

RESULTS AND DISCUSSION

Synthesis and Characterization of Stable and Purified Ag-peptide NPs in the PBS Buffer

We synthesized, purified and characterized the Ag NPs (11 nm in diameter), as described in Method.^{29, 41, 58} We then functionalized the surface of Ag NPs with the biocompatible peptides (CALNNK or CALNNE) to prepare positively and negatively charged Ag-peptide NPs (Figure 1A), respectively.^{56–58} Each peptide consists of 5 identical amino acids with the identical sequence (CALNN) and differs only a single amino acid (lysine and glutamic acid with pK_a of 10.8 and 4.0) at its C-terminus, which offers positive and negative charges at pH 4–10, respectively. We attached the peptide onto the Ag NPs by the interaction of the -SH of cysteine with the surface of Ag NPs to generate Ag-CALNNK and Ag-CALNNE NPs, respectively. These nearly identical peptides minimize the potential effects of variation of the surface functional groups (peptides) and sizes of the NPs on efflux function of BmrA and enable us to study the charge-dependent efflux function of BmrA in single live cells.

We purified the Ag-peptide NPs by thoroughly washing them with DI water using centrifugation. We characterized the surface charges of the purified Ag-peptide NPs by

measuring their surface zeta potentials (ζ) over time, showing (3.0 ± 0.2) and $-(11.9 \pm 0.9)$ mV for the Ag-CALNNK and Ag-CALNNE NPs, respectively (Figure 1B).⁵⁸ We characterized the sizes and shapes of the Ag-peptide NPs using high-resolution TEM (Figure 2A–B), showing spherical NPs with similar sizes of 11.5 ± 2.9 and 11.3 ± 2.5 nm in diameters for Ag-CALNNK NPs^{+ ζ} and Ag-CALNNE NPs^{– ζ} , respectively.

LSPR spectra of single Ag NPs characterized using DFOMS show that a majority of the Ag NPs are plasmonic blue NPs.^{29–30, 33, 41} As the surface of the Ag NPs are functionalized with the peptides, LSPR spectra of single Ag-peptide NPs shift from blue to the green and show plasmonic green NPs with peak wavelengths (λ_{max}) ranging from 488 to 546 nm (Figure 2C–D), which enables us to monitor the Ag-peptide NPs (functional peptides on the NPs) *in situ* in real time. The red-shift of the LSPR spectra of the single NPs is attributed to the decrease of their surface reflectivity and dielectric constant, and increase of their sizes.^{35–38} Notably, scattering intensity of single Ag-peptide NPs became about one order of magnitude lower than the Ag NPs, which makes it even more challenging to image single NPs in single live cells.

Our previous studies show that the LSPR spectra of smaller Ag NPs (e.g., 11 nm in diameter) show higher dependence upon their sizes, shapes, surface morphologies and roughness,^{25–26} and the distribution of LSPR spectra of single Ag NPs measured by DFOMS is always larger than the size distribution of the NPs measured by TEM and DLS.^{29–30, 33, 41} Notably, the Ag NPs have been incubated with an excess amount of peptides under shaking over night to achieve full functionalization of Ag NPs with the peptides and to prepare Ag-peptide NPs. Thus, the observation of the larger distribution of LSPR spectra of single Ag-peptide NPs measured by DFOMS than their size distribution measured by TEM and DLS is likely attributed to the high heterogeneity of plasmonic properties of the Ag NPs themselves, but not the heterogeneity of functionalized peptides on the surface of NPs.

We characterized the stability (non-aggregation) of positively and negatively charged Ag-peptide NPs in the PBS buffer for 24 h using DLS, which shows that their sizes remain essentially unchanged over 24 h (Figure 3). The sizes of the positively charged NPs measured by DLS are 14.1 ± 9.6 nm and 14.6 ± 3.0 nm at 0 and 24 h incubation with the PBS buffer. The sizes of the negatively charged NPs measured by DLS are 13.5 ± 3.0 nm and 14.0 ± 2.7 nm at 0 and 24 h incubation with the PBS buffer. The sizes of the NPs suspended in the PBS buffer measured by DSL are slightly larger than those measured by TEM (Figure 2A), which could be attributed to the solvation of the NPs in the solution and that the peptides on the surface of NPs could only be measured by DSL, but not by TEM. In this study, we used the sizes of NPs measured by TEM to present our results.

Real-time Imaging of Efflux Kinetics of Single Membrane Transporters in Single Live Cells

We used the distinctive LSPR spectra (colors) of single Ag-peptide NPs to identify single NPs over cellular membranes, debris and vesicles, which do not possess plasmonic properties and appear white under dark-field illumination. Therefore, LSPR spectra of single NPs enable us to identify and track the transport of single NPs inside and outside single live cells (WT and BmrA) using DFOMS. Dark-field optical images of the single live cells (WT and BmrA) (Figures 4 and S1) show cross-sections (hollow tubes, but not rods) of

single rod-like bacterial cells with 2 μm in length and 0.5 μm in width, and the membranes of single live cells above and below the focal plane are invisible under dark-field illumination, indicating that the focal plane (190 nm depth of field) of dark-field microscope enables us to image the thin-layer sections of single live cells with single NPs. The NPs appear to be on the membrane and stick outside the cells (Figures 4b-c and S1b-c), which is attributed to the higher scattering intensity of the NPs than the cell membrane and the sizes of the NPs and thickness of the cellular membrane (9 nm) are both under optical diffraction limit (~ 200 nm).

As the dark-field illumination passes through the cellular membrane to radiate the intracellular NPs, and scattering of intracellular NPs passes through the membrane to reach the detector, the cellular membrane absorbs and scatters photons. Thus, the intracellular NPs are dimmer and blurry and show lower scattering intensity than the NPs outside the cells (Figure 4b and S1b). In contrast, the scattering intensity of the extracellular NPs on the membrane includes the scattering intensity of both NPs and cellular membrane. Thus, they are brighter and show higher scattering intensity than intracellular NPs (Figure 4c and S1c). We have demonstrated that these distinctive features and approaches can be used to distinguish individual intracellular and extracellular NPs for study of efflux function of single membrane transporters in single live cells in real time.^{29–32, 43–44} We have also validated these approaches by imaging individual intracellular and extracellular NPs at the nanometer spatial resolution using TEM.³¹

Taken together, we have used the LSPR spectra and scattering intensity of single NPs to determine intracellular and extracellular NPs. The intracellular and extracellular NPs exhibit distinctive LSPR spectra. The intracellular NPs are dimmer and blurry and exhibit lower scattering intensity than the extracellular NPs (Figures 4b and S1b), while the extracellular NPs are brighter and show higher scattering intensity than intracellular NPs (Figures 4c and S1c). Furthermore, we used the LSPR spectra and scattering intensity of single NPs to characterize the size and number of single NPs, and monitor the peptides functionalized on the surface of the NPs *in situ* in real time as they are incubated with the cells and transported across the cellular membrane over time using the similar approaches as we reported previously.^{29–32, 43–44}

Notably, if the NPs were aggregated to become the larger NPs, we would have observed the red-shift of LSPR spectra and higher scattering intensity of the single NPs. If the peptides were released from the surface of the NPs, we would have observed the blue-shift of the single NPs. We did not observe either phenomenon, which demonstrate that the NPs are stable in the PBS buffer and in the cells over time and we have successfully detected single Ag-peptide NPs inside and outside of the single live cells over time to study the efflux function of single membrane transporter (BmrA) in single live cells.

Charge- and BmrA- Dependent Accumulation of Single Ag-peptide NPs in Single Live Cells

We studied the accumulation rates of positively and negatively charged Ag-peptide NPs in both WT and BmrA (devoid of BmrA) cells to determine the dependence of accumulation kinetics of intracellular NPs upon the charge of the NPs and the expression of BmrA in single live cells. The results in Figure 5 and Table 1 show the high dependence of

accumulation rate (slope of the plot over a given duration) of the NPs in single live cells upon the charge of NPs and cellular expression of BmrA. The positively charged NPs exhibit 4x higher accumulation rate in WT at 2 NPs/min than negatively charged NPs at 0.5 NPs/min (Figure 5A). Interestingly, both positively charged and negatively charged NPs show nearly identical accumulation rate in Δ BmrA (devoid of BmrA) at 2 NPs/min (Figure 5B), indicating that the type of their charges does not affect their entry into the cells, and negative charges of the NPs do not hinder them from going through negatively charged phospholipids membrane.

Plots of the number of positively charged NPs in WT (normal expression of BmrA) and Δ BmrA (devoid of BmrA) cells (Figure 6A) show the nearly identical accumulation rates at 2 NPs/min and the same amount of intracellular NPs at any given time over the course of the first hour incubation. This result shows the independent accumulation rate of positively charged NPs upon the expression of BmrA, and indicates that BmrA cannot extrude the positively charged NPs out of the cells (Figure 6A). If BmrA could extrude the positively charged NPs out of the cells, it would have led to the lower accumulation of the positively charged NPs in WT. The accumulation of the positively charged NPs in WT reaches a plateau after one hour incubation, while the NPs continue to be accumulated in Δ BmrA, which could be attributed to the lower accumulation capacity of the WT than Δ BmrA or possible induced efflux function of BmrA by the NPs. Thus, a slightly lower accumulation number of the positively charged NPs in WT than Δ BmrA were observed after the first hour of incubation.

In contrast, the number of negatively charged NPs accumulated in WT (normal expression of BmrA) is significantly lower than in Δ BmrA (devoid of BmrA) at any given time throughout 2 h incubation (Figure 6B), which shows that the accumulation rate of negatively charged NPs in WT at 0.5 NPs/min is 4x time lower than in Δ BmrA at 2 NPs/min, suggesting that the BmrA extrudes the negatively charged NPs out of the WT cells. We also observed the slightly decrease of the intracellular NPs in the WT after 70 min of incubation (Figure 6B-b), which could be attributed to the decrease of accumulation rate, as passive diffusion of the NPs into the cells reaches equilibrium due to the decrease of NP concentration gradient across the cellular membrane, while the efflux rate remains the same. Notably, the decrease of accumulation rate of negatively charged NPs in Δ BmrA was also observed after 70 min incubation (Figure 6B-a), which further confirms that the decrease of accumulation rate in WT after 70 min incubation is most likely attributed to the decrease of NP concentration gradient, but not the increase of the efflux rate.

It is worth noting that endocytosis, pinocytosis and exocytosis do not exist in prokaryotes (bacterial cells, *B. subtilis*) and they are not responsible for the transport of NPs in and out of the cells. Taken together, the results in Figures 5 and 6 indicate that the positively charged NPs cannot be extruded out of the cells by the BmrA membrane transporter, while the negatively charged NPs can. To our knowledge, the dependence of efflux function of BmrA upon the type of charges of the pump substrates has not yet been reported previously. Notably, the previous studies have showed that the BmrA can selectively extrude the positively charged fluorophores (e.g., Hoechst 33342) out of the cells.^{18, 29} These interesting findings suggest that the efflux function and mechanisms of BmrA for the NPs may be size-

dependent or it may differ from those of conventional antibiotics and drug molecules that are an order of magnitude smaller than the NPs. We are currently investigating the dependence of efflux kinetics of the charged NPs by BmrA in single live cells upon the sizes of the NPs, and we will report our finding in the near future.

Concentration-Dependent Accumulation of Single Ag-peptide NPs in Single Live Cells

To further determine how the NPs enter the live cells, we studied the dependence of accumulation of single NPs in single live cells upon concentration and charges of the NPs, and cellular expression of BmrA. For both positively and negatively charged NPs, the results in Figure 7 show that the number of intracellular NPs in both strains (WT, BmrA) highly depends upon NP concentrations and the number of intracellular NPs increases as the NP concentration increases. Interestingly, the number of positively charged NPs and their accumulation rates in both WT and BmrA are nearly proportional to the NP concentration (Figure 7A), showing independence of accumulation of positively charged NPs in single live cells upon the expression of BmrA. In contrast, the number of negatively charged NPs and their accumulation rates in BmrA are proportional to the NP concentration (Figure 7B-b), but those in the WT are not (Figure 7B-a), showing high dependence of accumulation of negatively charged NPs in single live cells upon the expression of BmrA and suggesting that BmrA extrude the negatively charged NPs out of the cells.

Notably, the passive diffusion rates of the NPs into the cells are driven by the concentration gradient across the cellular membrane, and they are proportional to the NP concentration. In contrast, the efflux of NPs out of the cells by the BmrA is an active process and the efflux rates are driven by the ATP hydrolysis upon its binding with NBD of the BmrA. Therefore, the accumulation of the negatively charged NPs in WT is not proportional to the NP concentration (Figure 7B-a).

Taken together, the results in Figure 7 suggest that the NPs, like antibiotics, most likely passively diffused into the cells. These results further demonstrate that both positively and negatively charged NPs can go through the cellular member and enter the cells. Notably,

BmrA cells are unable to extrude both positively and negatively charged NPs out of the cells, due to the absence of BmrA transporter, which leads to nearly identical accumulation rates of both positively and negatively charged NPs in single live cells (Figure 7b).

Furthermore, the BmrA cannot extrude the positively charged NPs out of the WT, leading to the same amount of the accumulation of positively charged NPs in both WT and BmrA at any given time over 2 h incubation (Figure 7A). In contrast, the BmrA transporter can extrude the negatively charged NPs out of the WT, leading to the lower accumulation of negatively charged NPs in WT than BmrA (Figure 7B).

Characterization of Viability of Single Cells

We characterized the viability of cells (WT, BmrA) incubated with the NPs throughout the duration of the experiments over 2 h using live/dead *bac*Light viability and counting assay, which uses SYTO9 nucleic acid stain and propidium iodide to detect live and dead cells, respectively.⁶² SYTO9 diffuses into live bacterial cells and leads to the green fluorescence ($\lambda_{\text{max}} = 520 \text{ nm}$), which enables the detection of viable cells. Notably, propidium iodide can

only enter into the dead bacterial cells due to the disintegration of the cellular membrane of the dead cells, and leads to red fluorescence ($\lambda_{\text{max}} = 610 \text{ nm}$), which enables the identification of the dead cells.

Representative optical images of the cells (WT) incubated with 1.4 nM positively charged NPs (Figure 8A-a) and negatively charged NPs (Figure 8B-a) for 2 h show that the cells with and without the NPs. Their fluorescence images show the green fluorescence, but not red fluorescence, indicating that the cells with and without NPs are alive (Figure 8b). We determine the number of live and dead cells and present the percentage of the viable cells by dividing the number of live cells with the total number of the cells. The results show that 94% of WT and 95% of BmrA cells incubated with the positively charged NPs over 2 h are alive, and 98% of either WT or BmrA cells incubated with the negatively charged NPs over 2 h are alive (Figure 8C), which are similar to what we observed previously using citrated Ag NPs.^{29, 43} Notably, the cells with the NPs are all alive. This result shows that both positively and negatively charged Ag-peptide NPs (1.4 nm) are biocompatible to the cells, and the NPs are well suited to serve as optical imaging probes for real-time study of charged-dependent efflux function of BmrA membrane transporter of single live cells. The results further demonstrate that accumulation of the NPs in single live cells is not attributed to the disintegration of cellular membrane due to the cell death, because the cells with the NPs are alive.

SUMMARY

In summary, we have successfully synthesized and characterized positively and negatively charged Ag-peptide NPs and used their distinctive LSPR spectra to identify and track single NPs and to study the charged-dependent efflux kinetics and function of BmrA in single live cells in real time. We found that a majority of the cells (94–98%) incubated with the NPs (1.4 nM) over 2 h are alive, which demonstrates that the NPs with a concentration up to 1.4 nM are highly biocompatible with the cells and can be used to directly study the efflux kinetics of BmrA in single live cells. We observed the high-dependent accumulation of single Ag-peptide NPs in single live cells (WT, BmrA) upon the charge and concentration of NPs, and cellular expression of BmrA. The accumulate rate (2 NPs/min) of positively charged NPs in WT and BmrA are nearly identical, showing independence upon the expression of BmrA and suggesting that BmrA do not extrude the positively charges NPs out of the cells. In contrast, the accumulation rate of negatively charged NPs in WT (0.5 NPs/min) is 4x lower than BmrA (2 NPs/min), suggesting that BmrA extrude the negatively charged NPs out of the cells, which leads to the lower accumulation in WT than in BmrA. Furthermore, the number of intracellular NPs in both strains (WT, BmrA) highly depends upon NP concentration and increases with the NP concentration, suggesting that the NPs enter the cells via passive diffusion, similar to conventional pump substrates (antibiotics). Taken together, this study shows that BmrA can extrude the negatively charged NPs, but not positively charged NPs. Notably, the previous studies have shown that BmrA can selectively extrude the positively charged fluorophores out of the cells,^{18, 29} suggesting that the efflux function and mechanisms of BmrA for the NPs may be size-dependent or it may differ from those conventional antibiotics that are an order of magnitude smaller than the NPs. These interesting findings represent the first study of charged-dependent efflux function of BmrA

in single live cells and demonstrate that single plasmonic NPs can serve as unique and powerful molecular imaging probes to study efflux functions of single membrane transporters in single live cells in real-time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Higgins CF. ABC Transporters: From Microorganisms to Man. *Annu Rev Cell Biol.* 1992; 8:67–113. [PubMed: 1282354]
2. Rees DC, Johnson E, Lewinson O. ABC Transporters: The Power to Change. *Nat Rev Mol Cell Biol.* 2009; 10:218–227. [PubMed: 19234479]
3. Schneider E, Eckey V, Weidlich D, Wiesemann N, Vahedi-Faridi A, Thaben P, Saenger W. Receptor-Transporter Interactions of Canonical ATP-Binding Cassette Import Systems in Prokaryotes. *Eur J Cell Biol.* 2012; 91:311–317. [PubMed: 21561685]
4. Cole SP. Multidrug Resistance Protein 1 (MRP1, ABCC1), a “Multitasking” ATP-Binding Cassette (ABC) Transporter. *J Biol Chem.* 2014; 289:30880–30888. [PubMed: 25281745]
5. Nigam SK. What Do Drug Transporters Really Do? *Nat Rev Drug Discov.* 2015; 14:29–44. [PubMed: 25475361]
6. Gottesman MM, Ambudkar SV, Xia D. Structure of a Multidrug Transporter. *Nat Biotechnol.* 2009; 27:546–547. [PubMed: 19513059]
7. Higgins CF. Abc Transporters: Physiology, Structure and Mechanism--an Overview. *Res Microbiol.* 2001; 152:205–210. [PubMed: 11421269]
8. Hung LW, Wang IX, Nikaido K, Liu PQ, Ames GF, Kim SH. Crystal Structure of the ATP-Binding Subunit of an ABC Transporter. *Nature.* 1998; 396:703–707. [PubMed: 9872322]
9. Locher KP, Lee AT, Rees DC. The E. Coli Btcd Structure: A Framework for ABC Transporter Architecture and Mechanism. *Science.* 2002; 296:1091–1098. [PubMed: 12004122]
10. Davidson AL, Chen J. ATP-Binding Cassette Transporters in Bacteria. *Annu Rev Biochem.* 2004; 73:241–268. [PubMed: 15189142]
11. Holland IB, Blight MA. ABC-ATPases, Adaptable Energy Generators Fuelling Transmembrane Movement of a Variety of Molecules in Organisms from Bacteria to Humans. *J Mol Biol.* 1999; 293:381–399. [PubMed: 10529352]
12. Ffrench B, Gasch C, O’Leary JJ, Gallagher MF. Developing Ovarian Cancer Stem Cell Models: Laying the Pipeline from Discovery to Clinical Intervention. *Mol Cancer.* 2014; 13:262. [PubMed: 25495823]
13. Morjani H, Aouali N, Belhoussine R, Veldman RJ, Levade T, Manfait M. Elevation of Glucosylceramide in Multidrug-Resistant Cancer Cells and Accumulation in Cytoplasmic Droplets. *Int J Cancer.* 2001; 94:157–165. [PubMed: 11668492]
14. Mortimer PG, Piddock LJ. A Comparison of Methods Used for Measuring the Accumulation of Quinolones by Enterobacteriaceae, *Pseudomonas Aeruginosa* and *Staphylococcus Aureus*. *J Antimicrob Chemother.* 1991; 28:639–653. [PubMed: 1663928]
15. Orelle C, Gubellini F, Durand A, Marco S, Levy D, Gros P, Di Pietro A, Jault JM. Conformational Change Induced by ATP Binding in the Multidrug ATP-Binding Cassette Transporter BmrA. *Biochemistry.* 2008; 47:2404–2412. [PubMed: 18215075]

16. Steel C, Wan Q, Xu XH. Single Live Cell Imaging of Chromosomes in Chloramphenicol-Induced Filamentous *Pseudomonas Aeruginosa*. *Biochemistry*. 2004; 43:175–82. [PubMed: 14705943]
17. Xu XHN, Brownlow WJ, Huang S, Chen J. Real-Time Measurements of Single Membrane Pump Efficiency of Single Living *Pseudomonas Aeruginosa* Cells Using Fluorescence Microscopy and Spectroscopy. *Biochem Biophys Res Commun*. 2003; 305:79–86. [PubMed: 12732199]
18. Ding F, Lee K, Vahedi-Faridi A, Huang T, Xu XHN. Design and Probing of Efflux Function of EGFP Fused ABC Membrane Transporters in Live Cells Using Fluorescence Spectroscopy. *Anal Bioanal Chem*. 2011; 400:223–235. [PubMed: 21336797]
19. Ding F, Lee KJ, Vahedi-Faridi A, Yoneyama H, Osgood CJ, Xu XHN. Design and Study of the Efflux Function of the EGFP Fused MexAB-OprM Membrane Transporter in *Pseudomonas Aeruginosa* Using Fluorescence Spectroscopy. *Analyst*. 2014; 139:3088–3096. [PubMed: 24781334]
20. Steinfelds E, Orelle C, Fantino JR, Dalmas O, Rigaud JL, Denizot F, Di Pietro A, Jault JM. Characterization of YvcC (BmrA), a Multidrug ABC Transporter Constitutively Expressed in *Bacillus Subtilis*. *Biochemistry*. 2004; 43:7491–7502. [PubMed: 15182191]
21. Bohren, CF., Huffman, DR. Absorption and Scattering of Light by Small Particles. Wiley; New York: 1983. p. 287-380.
22. Kelly KL, Coronado E, Zhao LL, Schatz GC. The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment. *J Phys Chem B*. 2003; 107:668–677.
23. Kreibig, U., Vollmer, M. Optical Properties of Metal Clusters. Springer; Berlin: 1995. p. 14-123.
24. Mie G. Beitrag Zur Optik Trüber Medien, Speziell Kolloidaler Metallösungen. *Annu Phys*. 1908; 25:377–445.
25. Nallathamby PD, Huang T, Xu XHN. Design and Characterization of Optical Nano Rulers of Single Nanoparticles Using Optical Microscopy and Spectroscopy. *Nanoscale*. 2010; 2:1715–1722. [PubMed: 20820702]
26. Huang T, Xu XHN. Synthesis and Characterization of Tunable Rainbow Colored Silver Nanoparticle Solutions Using Single-Nanoparticle Plasmonic Microscopy and Spectroscopy. *J Mater Chem*. 2010; 20:9867–9876. [PubMed: 22707855]
27. Mulvaney P. Surface Plasmon Spectroscopy of Nanosized Metal Particles. *Langmuir*. 1996; 12:788–800.
28. Bingham JM, Henry AI, Blaber MG, Schatz GC, Van Duyne RP. LSPR Imaging of Triangular Nanoprisms: Correlating Plasmon Spectra with Structure Using Electrodynamics for Plasmon Lifetime Analysis. *J Phys Chem C*. 2012; 116:393–403.
29. Lee KJ, Browning LM, Huang T, Ding F, Nallathamby PD, Xu XHN. Probing of Multidrug ABC Membrane Transporters of Single Living Cells Using Single Plasmonic Nanoparticle Optical Probes. *Anal Bioanal Chem*. 2010; 397:3317–3328. [PubMed: 20544182]
30. Nallathamby PD, Lee KJ, Desai T, Xu XHN. Study of Multidrug Membrane Transporters of Single Living *Pseudomonas Aeruginosa* Cells Using Size-Dependent Plasmonic Nanoparticle Optical Probes. *Biochemistry*. 2010; 49:5942–5953. [PubMed: 20540528]
31. Xu XHN, Brownlow WJ, Kyriacou SV, Wan Q, Viola JJ. Real-Time Probing of Membrane Transport in Living Microbial Cells Using Single Nanoparticle Optics and Living Cell Imaging. *Biochemistry*. 2004; 43:10400–10413. [PubMed: 15301539]
32. Xu XHN, Chen J, Jeffers RB, Kyriacou SV. Direct Measurement of Sizes and Dynamics of Single Living Membrane Transporters Using Nano-Optics. *Nano Lett*. 2002; 2:175–182.
33. Nallathamby PD, Lee KJ, Xu XHN. Design of Stable and Uniform Single Nanoparticle Photonics for in Vivo Dynamics Imaging of Nanoenvironments of Zebrafish Embryonic Fluids. *ACS Nano*. 2008; 2:1371–1380. [PubMed: 19206304]
34. Nallathamby PD, Xu XHN. Study of Cytotoxic and Therapeutic Effects of Stable and Purified Silver Nanoparticles on Tumor Cells. *Nanoscale*. 2010; 2:942–952. [PubMed: 20648292]
35. Huang T, Browning LM, Xu XHN. Far-Field Photostable Optical Nanoscopy (PHOTON) for Real-Time Super-Resolution Single-Molecular Imaging of Signaling Pathways of Single Live Cells. *Nanoscale*. 2012; 4:2797–2812. [PubMed: 22331098]

36. Huang T, Nallathamby PD, Gillet D, Xu XHN. Design and Synthesis of Single Nanoparticle Optical Biosensors for Imaging and Characterization of Single Receptor Molecules on Single Living Cells. *Anal Chem.* 2007; 79:7708–7718. [PubMed: 17867652]
37. Huang T, Nallathamby PD, Xu XHN. Photostable Single-Molecule Nanoparticle Optical Biosensors for Real-Time Sensing of Single Cytokine Molecules and Their Binding Reactions. *J Am Chem Soc.* 2008; 130:17095–17105. [PubMed: 19053435]
38. Huang T, Xu XHN. Multicolored Nanometer-Resolution Mapping of Single Protein-Ligand Binding Complex Using Far-Field Photostable Optical Nanoscopy (PHOTON). *Nanoscale.* 2011; 3:3567–3572. [PubMed: 21633732]
39. Browning LM, Lee KJ, Huang T, Nallathamby PD, Lowman J, Xu XHN. Random Walk of Single Gold Nanoparticles in Zebrafish Embryos Leading to Stochastic Toxic Effects on Embryonic Developments. *Nanoscale.* 2009; 1:138–152. [PubMed: 20644873]
40. Lee KJ, Browning LM, Nallathamby PD, Desai T, Cherukui P, Xu XHN. *In Vivo* Quantitative Study of Size-Dependent Transport and Toxicity of Single Silver Nanoparticles Using Zebrafish Embryos. *Chem Res Toxicol.* 2012; 25:1029–1046.
41. Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XHN. *In Vivo* Imaging of Transport and Biocompatibility of Single Silver Nanoparticles in Early Development of Zebrafish Embryos. *ACS Nano.* 2007; 1:133–143. [PubMed: 19122772]
42. Lee KJ, Nallathamby PD, Browning LM, Desai T, Cherukui P, Xu XHN. Single Nanoparticle Spectroscopy for Real-Time *in Vivo* Quantitative Analysis of Transport and Toxicity of Single Nanoparticles in Single Embryos. *Analyst.* 2012; 137:2973–2986. [PubMed: 22563577]
43. Browning LM, Lee KJ, Cherukuri PK, Nallathamby PD, Warren S, Jault JM, Xu XHN. Single Nanoparticle Plasmonic Spectroscopy for Study of Efflux Function of Multidrug ABC Membrane Transporters of Single Live Cells. *RSC Advances.* 2016; 6:36794–36802. [PubMed: 27570617]
44. Kyriacou SV, Brownlow WJ, Xu XHN. Using Nanoparticle Optics Assay for Direct Observation of the Function of Antimicrobial Agents in Single Live Bacterial Cells. *Biochemistry.* 2004; 43:140–147. [PubMed: 14705939]
45. Conseil G, Rothnie AJ, Deeley RG, Cole SP. Multiple Roles of Charged Amino Acids in Cytoplasmic Loop 7 for Expression and Function of the Multidrug and Organic Anion Transporter MRP1 (ABCC1). *Mol Pharmacol.* 2009; 75:397–406. [PubMed: 19015228]
46. Eckey V, Weidlich D, Landmesser H, Bergmann U, Schneider E. The Second Extracellular Loop of Pore-Forming Subunits of ATP-Binding Cassette Transporters for Basic Amino Acids Plays a Crucial Role in Interaction with the Cognate Solute Binding Protein(s). *J Bacteriol.* 2010; 192:2150–2159. [PubMed: 20154136]
47. El-Sheikh AA, van den Heuvel JJ, Krieger E, Russel FG, Koenderink JB. Functional Role of Arginine 375 in Transmembrane Helix 6 of Multidrug Resistance Protein 4 (MRP4/ABCC4). *Mol Pharmacol.* 2008; 74:964–971. [PubMed: 18612080]
48. Haimeur A, Deeley RG, Cole SP. Charged Amino Acids in the Sixth Transmembrane Helix of Multidrug Resistance Protein 1 (MRP1/ABCC1) Are Critical Determinants of Transport Activity. *J Biol Chem.* 2002; 277:41326–41333. [PubMed: 12186871]
49. Iram SH, Cole SP. Expression and Function of Human MRP1 (ABCC1) Is Dependent on Amino Acids in Cytoplasmic Loop 5 and Its Interface with Nucleotide Binding Domain 2. *J Biol Chem.* 2011; 286:7202–7213. [PubMed: 21177244]
50. Lagas JS, Sparidans RW, van Waterschoot RA, Wagenaar E, Beijnen JH, Schinkel AH. P-Glycoprotein Limits Oral Availability, Brain Penetration, and Toxicity of an Anionic Drug, the Antibiotic Salinomycin. *Antimicrob Agents Chemother.* 2008; 52:1034–1039. [PubMed: 18195061]
51. Müller M, Mayer R, Hero U, Keppler D. ATP-Dependent Transport of Amphiphilic Cations across the Hepatocyte Canalicular Membrane Mediated by MDR1 P-Glycoprotein. *FEBS Lett.* 1994; 343:168–172. [PubMed: 7909523]
52. Zhang DW, Gu HM, Situ D, Haimeur A, Cole SP, Deeley RG. Functional Importance of Polar and Charged Amino Acid Residues in Transmembrane Helix 14 of Multidrug Resistance Protein 1 (MRP1/ABCC1): Identification of an Aspartate Residue Critical for Conversion from a High to

Low Affinity Substrate Binding State. *J Biol Chem.* 2003; 278:46052–46063. [PubMed: 12954620]

53. Aguilar C, Vlamakis H, Losick R, Kolter R. Thinking About *Bacillus Subtilis* as a Multicellular Organism. *Curr Opin Microbiol.* 2007; 10:638–643. [PubMed: 17977783]
54. Quentin Y, Fichant G, Denizot F. Inventory, Assembly and Analysis of *Bacillus Subtilis* ABC Transport Systems. *J Mol Biol.* 1999; 287:467–484. [PubMed: 10092453]
55. Völker U, Hecker M. From Genomics Via Proteomics to Cellular Physiology of the Gram-Positive Model Organism *Bacillus Subtilis*. *Cell Microbiol.* 2005; 7:1077–1085. [PubMed: 16008575]
56. Doty RC, Tshikhudo TR, Brust M, Fernig DG. Extremely Stable Water-Soluble Ag Nanoparticles. *Chem Mater.* 2005; 17:4630–4635.
57. Wang Z, Levy R, Fernig DG, Brust M. The Peptide Route to Multifunctional Gold Nanoparticles. *Bioconjug Chem.* 2005; 16:497–500. [PubMed: 15898714]
58. Lee KJ, Browning LM, Nallathamby PD, Xu XHN. Study of Charge-Dependent Transport and Toxicity of Peptide-Functionalized Silver Nanoparticles Using Zebrafish Embryos and Single Nanoparticle Plasmonic Spectroscopy. *Chem Res Toxicol.* 2013; 26:904–917. [PubMed: 23621491]
59. Gebhart SC, Thompson RC, Mahadevan-Jansen A. Liquid-Crystal Tunable Filter Spectral Imaging for Brain Tumor Demarcation. *Appl Opt.* 2007; 46:1896–1910. [PubMed: 17356636]
60. Huang T, Cai W, Elsayed-Ali HE, Xu XHN. High-Throughput Ultrasensitive Characterization of Chemical, Structural and Plasmonic Properties of EBL-Fabricated Single Silver Nanoparticles. *Nanoscale.* 2012; 4:380–385. [PubMed: 22117236]
61. Ocaktan A, Yoneyama H, Nakae T. Use of Fluorescence Probes to Monitor Function of the Subunit Proteins of the MexA-MexB-OprM Drug Extrusion Machinery in *Pseudomonas Aeruginosa*. *J Biol Chem.* 1997; 272:21964–21969. [PubMed: 9268332]
62. Berney M, Hammes F, Bosshard F, Weilenmann HU, Egli T. Assessment and Interpretation of Bacterial Viability by Using the Live/Dead BacLight Kit in Combination with Flow Cytometry. *Appl Environ Microbiol.* 2007; 73:3283–3290. [PubMed: 17384309]

Figure 1. Synthesis and characterization of positively and negatively charged Ag-peptide NPs. **(A)** The Ag-peptide NPs, **(a)** Ag-CALNNK NPs⁺ ζ and **(b)** Ag-CALNNE NPs⁻ 4ζ , are synthesized by the interaction of the thiol group (-SH) of cysteine with the surface of Ag NPs via the replacement reaction of citrate with peptides to functionalize Ag NPs with the give type of peptides, either CALNNK or CALNNE, respectively. **(B)** Plots of zeta potentials of the NPs versus time show that their zeta potentials remain unchanged over time at: (a) 3.0 ± 0.2 and (b) -11.9 ± 0.9 mV, respectively.⁵⁸

Figure 2.

Characterization of size and optical properties of Ag-peptide NPs. (A) Representative TEM images; (B) histograms of the size distribution; (C) dark-field optical images, and LSPR spectra of individual (a) Ag-CALNNK NPs^{+ζ} and (b) Ag-CALNNE NPs^{-4ζ} show spherical shaped NPs with an average diameter of (a) 11.5 ± 2.9 nm and (b) 11.3 ± 2.5 nm, and plasmon green NPs with peak wavelengths (λ_{max}) and full-width-of-half-maximum (FWHM) at (a) (i) 488 (62), (ii) 540 (74) and (iii) 541 (92) nm; and (b) (i) 497 (30), (ii) 517 (47) and (iii) 546 (81) nm, respectively. Scale bars are 20 nm in (A) and 2 μm in (C). The

scale bar in (C) shows the distance between individual NPs, but not their sizes because of optical diffraction limit.

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Figure 3.

Study of stability (non-aggregation) of positively and negatively charged Ag-peptide NPs: (a) Ag-CALNNK NPs^{+ζ} and (b) Ag-CALNNE NPs^{-4ζ}, in the PBS buffer (0.5 mM phosphate buffer, 1.5 mM NaCl, pH = 7.0) over 24 h. Size distribution measured by DLS ($C_{\text{NPs}} = 1.4 \text{ nM}$) of (A) Ag-CALNNK NPs^{+ζ} show their average diameters of (i) $14.1 \pm 9.6 \text{ nm}$ and (ii) $14.6 \pm 3.0 \text{ nm}$; (B) Ag-CALNNE NPs^{-4ζ} show their average diameters of (i) $13.5 \pm 3.0 \text{ nm}$ and (ii) $14.0 \pm 2.7 \text{ nm}$, at (i) 0 and (ii) 24 h, respectively.

Figure 4.

Real-time imaging of single intracellular and extracellular Ag-peptide NPs with single live cells. Representative CCD images of WT cells incubated with 1.4 nM (A) Ag-CALNNK NPs⁺⁵ and (B) Ag-CALNNE NPs⁻⁴⁵ show (a) a full image of single cells with (b) intracellular and (c) extracellular NPs. The plasmonic colors of the NPs in (b) and (c) were characterized using their LSPR spectra similar to those in Figure 2D and their pseudo colors were added into the images based upon their spectra. The scale bar is 4 μm in (a) and 2 μm in (b) and (c).

Figure 5.

Study of the charge-dependent accumulation kinetics of single positively and negatively charged Ag-peptide NPs ($C_{\text{NPs}} = 1.4 \text{ nM}$) in single live cells. Plots of number of intracellular (a) Ag-CALNNK NPs^{+ζ}, and (b) Ag-CALNNE NPs^{-4ζ} in (A) WT and (B) BmrA cells versus incubation time show the high dependence of accumulation kinetics of NPs upon the charge of the NPs. The points represent the sum of three repeated experiments and the lines are added to guide the trend. At each point (every 20 min), 300 cells are imaged at 15 cells/min over time.

Figure 6.

Study of the BmrA-dependent accumulation kinetics of single positively and negatively charged Ag-peptide NPs ($C_{\text{NPs}} = 1.4 \text{ nM}$) in single live cells. Plots of number of intracellular (A) Ag-CALNNK NPs^{+ζ} and (B) Ag-CALNNE NPs^{-4ζ} in (a) BmrA (devoid of BmrA) and (b) WT (normal expression of BmrA) cells versus incubation time show the independent accumulation kinetics of positively charged Ag-peptide NPs, and the high dependent accumulation kinetics of negatively charged Ag-peptide NPs upon the cellular expression of BmrA. The points represent the sum of three repeated experiments and the lines are added to guide the trend. At each point (every 20 min), 300 cells are imaged at 15 cells/min over time.

Figure 7.

Study of the concentration-dependent accumulation kinetics of single positively and negatively charged Ag-peptide NPs in single live cells. Plots of number of intracellular NPs in (a) WT and (b) BmrA cells that were incubated with (i) 1.4 and (ii) 0.7 nM of (A) Ag-CALNNK NPs⁺ and (B) Ag-CALNNE NPs⁻ for 2 h show high dependent accumulation kinetics of the NPs upon the NP concentration. The points represent the sum of three repeated experiments and the lines are added to guide the trend. At each point (every 20 min), 300 cells are imaged at 15 cells/min over time.

Figure 8.

Characterization of the viability of single cells using live/dead *bacLight* viability and counting assay. (a) Dark field optical image and (b) fluorescence image of single cells (WT), incubated with 1.4 nM (A) Ag-CALNNK NPs^{+ζ} and (B) Ag-CALNNE NPs^{-4ζ} over the duration of each experiment for 2 h, show that the cells with or without intracellular NPs emit the green fluorescence of SYTO9 ($\lambda_{\text{max}} = 520 \text{ nm}$), but not red fluorescence, indicating viable cells. (C) Plots of percentage of viable cells incubated with (a) Ag-CALNNK NPs^{+ζ} and (b) Ag-CALNNE NPs^{-4ζ} for 2 h, for WT and ΔBmrA cells, show that (a) 94–95% and (b) 98% of the cells are alive, respectively. The scale bar is 4 μm in (A–B). More than 100 of cells are imaged for each experiment.

Study of Charged-Dependent Accumulation Rates and Number of Intracellular Single Ag-peptide NPs in Single Live Cells

Table 1

NPs	C (mM)	Diameter (nm) ^a	Zeta Potential (mV)	Rate (NPs min ⁻¹) ^b		Number of Intracellular NPs ^c	
				WT	BmrA	WT	BmrA
Ag-CALNNK NP- ζ	1.4	11.5 $\pm$ 2.9	2.9 $\pm$ 0.1	2.0	2.4	183	232
Ag-CALNNE NP- ζ	1.4	11.3 $\pm$ 2.5	-11.9 $\pm$ 0.9	0.5	2.3	31	203

^aDiameters of NPs measured by TEM;^bAccumulation rates (slopes of plots) of the NPs in 300 live cells over 120 min (Figure 5);^cNumber of intracellular NPs accumulated in 300 live cells at 120 min incubation